Callus initiation and subculture of *Taxus chinensis*

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Abstract Conditions have been established for the callus initiation and subculture of *T. chinensis*. The calli were induced by the explants cultured first on the medium MS supplemented with 1.0 mg/L 2,4-D, 2g/L CH, and 25g/L sucrose, then on The medium: MS+1.0 mg/L NAA+0.5 mg/L BA+2 g/L CH+25 g/L sucrose. When the callus was subcultured and tamed several times, it could grow fast and stable on the medium: MS+0.2mg/L 2,4-D+0.5 mg/L NAA+0.5 mg/L BA+2 g/LCH+25 g/L sucrose. The contamination of explants was a result of endophytic microbes of *T. chinensis*. This could be avoided by adopting the tender shoots 3-5 cm long collected in early spring as the source of explants. The browning of the cultures could be prevented and controlled by means of the selection of a suitable explants, hormonal regime in the medium, culture methods and the use of antioxidants.

Key words: *Taxus chinensis*, tissue culture, taxol.

Abbreviations: 2,4-D:2,4-dichlorphenoxyacetic acid; NAA-α : naphthaleneacetic acid; BA-6: benzylaminopurine; CH: casien enzymatic hydrolysate; GA_3: gibberellic acid; Zt: zeatin.

Introduction

Taxol is a complex diterpenoid secondary product of the genus *Taxus* that was approved for treatment against ovarian and breast cancers and shows promise against other cancers. Due to the relative scarcity of the few natural resources and the low yield of taxol, the supply of taxol is restricted, limiting expansion of clinical trials and treatment availability. Interest in alternative methods for taxol production has been intensifying. Cell culture provide a convenient system for studying the biosynthesis of taxol. It may be a viable alternative for taxol product (Jaziri M. *et al* 1996).

Taxus chinensis (Pliger Rehd) is a slow growing evergreen tree distributed in China. Many reports on taxol tissue and cell cultures have been published, but few of them are about *T. chinensis* tissue culture. The contamination of explants and culture browning are observed frequently in *Taxus* tissue culture (Jaziri M. *et al*. 1996). In the course of our work, we have experimented with conditions to allow initiation and subculture of the callus from *T. chinensis*. This paper describes conditions which have been successful in avoiding and controlling the explant contamination and callus culture browning. The information provides a support for the further research.

Received: 1998-08-25

Material and methods

Plant materials

Tender shoots 3~5 cm in length of *T. chinensis* growing in Hubei were obtained in early spring, summer and autumn, and stored in plastic bag sealed at 4℃.

Media preparations

Initiation medium:

I_1: MS+1.0 mg/L 2,4-D
I_2: B5+1.0 mg/L 2,4-D
I_3: MS+1.0 mg/L2,4-D+0.5mg/L NAA
I_4: MS+1.0 mg/L NAA
I_5: MS+1.0 mg/L NAA+0.5 mg/L BA

Subculture medium:

S_1: MS+1.0 mg/LNAA
S_2: MS+1.0 mg/L NAA+0.5 mg/L BA
S_3: MS+0.5 mg/L NAA+0.5 mg/L BA

All the aforesaid initiation media and subculture media were supplemented with 25 g/L sucrose, 2g casien enzymatic hydrolysate, 5 mL 20% $Na_2S_2O_3$, 5 mg ascorbic acid and 7.5g agar every litre, with pH adjusted to 5.5~5.8 prior to autoclaving. 25 mL of medium was aliquoted in each 50 mL Erlenmeyer flask. All the media were autoclaved 15 min at 121℃.

The callus initiation of *T. chinensis*

Shoots of *T. chinensis* were washed with running water, immersed in 0.1% mercuric chloride ($HgCl_2$) with 1~2 drops of Tween 80/100 mL gently shaken for about 6~8 min, then rinsed 5 times with sterile distilled

water, 1 time with 0.1g/L ascorbic acid (sterilized by filtering through 0.22 μm sterile filters), and aseptically dissected into explants 1 cm in length. Half of each leaf of the shoots was crosscut. Explants were placed on the medium (3 explants per Erlenmeyer flask, 60 bottles per initiation medium), and incubated in darkness, at 25+1℃.

Subculture of the callus cultures

Initially, calli were cultured and subcultured in the same initiation medium for 1~2 times at 2~3 weeks interval, and then the calli mixed evenly were transferred to different subculture media at 3 weeks interval, 8 Erlenmeyer flask per medium, approx. 1g callus per flask. The incubation conditions were as mentioned above. On the 30th day of the subculture, the calli were determined and changed into the corresponding value on the basis of 1g inoculation amount. The mean of 8 duplications was taken as the callus growth.

Results

The disposal of the explant contamination

The shoots of *T. chinensis* collected in different season were sterilized and inoculated as mentioned methods above. More than 80% explants from the tender shoots collected in early spring were not contaminated, while the explants collected in summer or autumn were done (especially the fugal contamination) partly on the 5th day after the inoculation and wholly on the 10th day because of the high level of endophytic microbes in the shoots.

According to the Gibson's (1993) method, 3 alternative stringent sterilization regimes were employed as follows:

a. A stringent initial treatments used: a 30~45 seconds 10% sodium hypochlorite (NaClO) dip, followed by a 4~12 hr incubation at 4℃, and rinsing 5 times with sterile distilled water prior to cutting into 1 cm section and inoculating.

b. The shoots were washed with soap solution, followed by a 30~45 s 95% ethanol dip, then immersed in a solution of 2% NaClO containing 2 drops of Tween 80 per 100 mL, and stirred on the rotatory shakers at 110r/min for 15 min. After rinsing 5 times with flamed following a 95% ethanol dip, half of each leaf was crosscut, and then the shoots were dissected into explants 1 cm length. Explants were placed onto the media.

c. The shoots were first dipped into 75% ethanol for 1 min, then 0.5% mercuric chloride (containing 2 drops of Tween 80/100 mL) for 10 min, and 2% NaClO (containing 2 drops of Tween 80/100 mL) for 15 min. After rinsing 5 times with sterile distilled water, half of each leaf was crosscut and shoots were cut into 1 cm section. The explants were inoculated onto the media.

Although the explants were rigorously sterilized in the above 3 different regimes, the microbial contamination still occurred in the first week after inoculation. Especially in the sterilization protocol b, some explants grew greensick and withered on the second day after the inoculation. Over 80% expalnts were still contaminated, and all the explants withered in the 2nd week after inoculation. Some expalnts also withered in protocol a and c. The shoots collected in summer and autumn were sterilized rigorously, even necrotic, many explant contamination still occurred. However, the explants from the tender shoots collected in spring were seldom contaminated through ordinary sterilization. The results indicated that the temperature in summer or autumn was higher, microbes were active, and there were some endophytic microbes in the shoots. This kind of shoots cannot be used as explants.

The effects of different media on callus initiation

T. chinensis is a species of slow-growing gymnosperm, the cultures of *T. chinensis* are liable to become brown coloured during the initial culture phase. To get the best callus initiation, 5 initiation media were design according to the related literature's. Initial callus induction occurred from the cut surface of the stems and leaves. However, a majority of the callus appeared to originate from the cambium of the stem and petiole which resulted in the expanding, splitting and/or peeling of those area due to the growing callus.

Table 1. Initiation of *T. chinensis* on various media

Medium	The number of explants without contamination	The number of explants producing callus	Percentage of callus initiation (%)
I_1	158	132	83.5
I_2	161	133	82.6
I_3	157	108	68.8
I_4	143	93	65.0
I_5	147	92	62.6

The callus was induced on different media, and all the callus initiation percentages on the 30th day after inoculation were 60% (Table. 1). MS medium is commonly used in plant tissue culture, but B_5 medium is the best basic medium for callus induction of *Taxus* sp. according to the literatures, I_1 and I_2 media were respectively MS and B_5 supplemented with the same components. The percentages of the callus initiation on I_1 and I_2 were respectively 83.5%, 82.5% and very close. The results demonstrated that both of MS and B_5 were good for callus of *T. chinesis*. The percentage of the callus initiation in the hormonal regimes of 1.0 mg/L 2,4-D or only 1.0 mg/L 2,4-D were higher than that of 1.0 mg/L NAA or only 1.0 mg/L NAA. Further-

more, the callus initiation in the hormonal regime of 2,4-D occurred earlier 2-3 days.

The control of the browning of the callus cultures. The browning of the callus culture appeared often in the callus initiation and subculture of *Taxus* sp. The callus cultures of *T. chinensis* secreted red/brown colored exudates visible either on the callus itself in the culture medium or in both, which resulted in the substantial growth declining, even the necroses of the callus cultures. To prevent the browning became the dominant factor in the successful callus initiation and subculture. The experiments indicated that the browning was relaxed by adding 5 mg/L ascorbic acid and 5 mg/L 20% $Na_2S_2O_3$, could not be controlled thoroughly. Finally the browning was prevented from through rinsing the shoots with 0.5 mg/L sterile ascorbic acid prior to the cut and inoculation, and dripping the explant with suitable antioxidant during the culture period at some day intervals.

The selection of the explants and suitable incubation conditions such as hormonal regime are usually the effective precaution measures against the browning, The results demonstrated that 2,4-D is good for the callus initiation, but liable to the browning of the callus cultures. So the callus cultures were first subcultured on the same initiation medium 1-2 times, and then transferred onto the medium supplemented with NAA, the browning could be greatly relaxed. Selecting the tender shoots 3~5 cm in length collected in earlier spring as the source of explants could also avoid the browning.

The experiment demonstrated that the calli should be transferred with explants when they were smaller 0.5-1 cm in diameter. Otherwise they would be browned easily when isolated from the explants too early. In the main the red/brown colored exudates did not occurred by the choice of explants suitable in combination with the use of antioxdants and other measures.

The callus subculture

As Table 2 shows, the best callus growth was got on the S_3: MS+0.5 mg/L NAA+0.5 mg/L BA.

Table 2. The callus growth on the different subculture media

Medium	S1	S2	S3
Callus growth(g. FW)	3.5	3.6	3.8

When callus was subcultured and tamed several times on this medium, the callus growth speed were greatly improved. Considering 2,4-D could promote cell division more effectively, 0.2 mg/L 2,4-D was add to the subculture medium. The callus grew fast and stable on the medium: MS+0.2 mg/L 2,4-D + 0.5 mg/L NAA + 0.5 mg/L BA + 2 g/L CH+ 25 g/L sucrose.

Discussion

The callus initiation and subculture with success is the first step in the taxol biosynthesis regulation in cell cultures of *T. chinensis*. The characteristics such as slow growth and gymnosperm make it more difficult. The contamination of the plant material is the common problem which was first met with in *Taxus* tissue culture. The experimental results demonstrated that the contamination was a result of the endophytic microbes of. *T chinensis*. The contamination could be avoided by adopting the tender shoots in early spring as the source of explant. Wheeler (1992) had noted the fact that taxol and taxane content appears to be under epigenetic, environmental and physiological controls. Some studies have reported that some fungal endophytes, which could produce taxol and related components, was isolated from *Taxus* sp. (Stierle *et al.* 1993; Qiu *et al* 1994; Strobel *et al* 1996) The authors suggested that taxol may be one kind of allelochemicals from *Taxus* sp., which inhibited *Taxus sp.* and microbes, and so this kinds of gymnosperm grow very slowly. This indicated to us that microbial elicitors may be the effective factor in taxol biosynthesis.

The red/brown colored exudates secreted by the callus cultures give rise to the substantial growth declines , even the necroses of callus cultures. The problem was successfully solved by means of the selection of suitable explants, hormonal regime in medium, culture methods and the use of antioidants.

The browning is a common problem in the callus and subculture process of wood plants. Now it is generally acknowledged that there are many phenolic compounds in the cells of wood plants, which could changed into quinoid by the function of polyphenol oxidase. In nature, the phenolic compounds and quinoids in the cells are in dynamic equilibrium, the PPO were inhibited, the quinoid stay at lower level. When the tissues are cut and cultured in vitro, the air will invade through the wounds, stimulating the activity of PPO, which catalyzed the phenolic compounds oxidized into quinoids quickly. The quinoid soon spreads into the medium and is stored, inhibiting the activities of many other enzymes in the cell, damaging the normal metabolism and finally, slowing down grow processes (Chen 1986).

In the author's opinion, the main inducing factors of the browning phenomenon in the callus culture are the plant material and the hormone composition in medium. The developmental stage and physiological state of the plant materials are internal cause of browning. We can determine from the literatures on tissue cultures of *Taxus* that the browning phenomenon seldom occurred when the embryo was used as

explant. The embryo is at the earliest developmental stage, which determined it to be the best materials for plant tissue culture. Gibson *et al* (1993). have proved that the age of the tree has no influence on the induction of callus tissue. Xia *et al* (1996) held that the young stems in growing season are good explants. In our experiment, we selected the young stem that had just grown to 3-5 cm in spring as materials, so the browning process will be inhibited. Using the material in summer and autumn as explant will need strict sterilizing process because of the invasion of microbe into the plant material. However the sterilizing process may damage the material, stimulate the internal PPO, or the sterilizing agent may remain in the material. All of these will induce the browning processes. Further researches are needed for this. The hormone composition in the medium is the external cause of the browning. Although 2,4-D is a hormone which could effectively induce the dedifferentiation in plant tissue culture, it may result in the browning of the cultures and be detrimental to the callus growth that the tissue cultures remain in the medium with high concentration of 2,4-D after the explant has differentiated. The explant after differentiation should be subcultured on the medium free or lower content of 2,4-D, or the medium just with other hormones be used for the callus initiation, for example, the medium for *T. cuspidata* as: MS+0.5 mg/L Zt+1.0mg/L NAA+2.0mg/L GA_3 +20g/L sucrose (Xia, et al.). This experiment demonstrated that it was beneficial to the callus growth that the explant after differentiation was subcultured on the medium without 2,4-D.

Acknowledgments

The authors thank Dr. Marcus J Darwent, a visiting scientist, for his revision of this paper.

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(**Responsible Editor**: Chai Ruihai)